Germline and Somatic Mutations of the *INK4a-ARF* Gene in a Xeroderma Pigmentosum Group C Patient

N. Soufir, M. Ribojad,* T. Magnaldo,† O. Thibaudeau,‡ G. Delestaing,‡ L. Daya-Grosjean,† J. Rivet,§ A. Sarasin,† and N. Basset-Seguin‡

Service de Biochimie-Génétique, Hôpital Bichat-Claude Bernard, Paris, France; *Service de, Paris Dermatologie Hôpital Saint-Louis, Paris France; †Molecular Genetics Laboratory, UPR42, CNRS, Villejuif, France; ‡Institut de Recherche sur la Peau, Inserm U 532, Hôpital Saint-Louis, Paris, France; §Service d'Anatomopathologie, Hôpital Saint-Louis, Paris, France

Xeroderma pigmentosum is an inheritable autosomal recessive DNA repair deficient syndrome characterized by a high predisposition to skin cancers. An elevated proportion of tumors from xeroderma pigmentosum patients harbor ultraviolet-induced mutations (CC:GG > TT:AA tandem transitions) of the p53 and/or the *INK4a-ARF* genes. Here, we report the clinical and molecular features of a 12 y old xeroderma pigmentosum patient who, in addition to severe cutaneous clinical symptoms, also had three unusual tumors, a mediastinal lymphoblastic lymphoma, an atypical fibroxanthoma, and an epithelioid hemangioma. Single strand conformation polymorphism and sequencing analysis of the p53 and *INK4a-ARF* genes were carried out in DNA from normal skin and different tumors (four actinic keratosis, two microinvasive squamous cell carcinomas, one basal cell carcinoma, and one atypical fibroxanthoma) from the patient. After characterization of the xeroderma pigmentosum C complementation group, we found unexpectedly that this patient also carried a germline mutation of the *INK4a-ARF* locus affecting the $p16^{INK4A}$ reading frame. Three different somatic mutations that all harbor the signature of ultraviolet light (two of $p16^{INK4A}$ and one of p53) were also detected in the basal cell carcinoma. We hypothesize that the germline mutation of $p16^{INK4A}$, in association with the nucleotide excision repair defect, could explain the patient's unusual phenotype. Furthermore, this study confirms that concomitant somatic mutations of *INK4a-ARF* and p53 occur in some xeroderma pigmentosum associated tumors, and seem to accumulate during tumor progression rather than the initiation step. ***Key words: lymphoma/mutation/$p14^{ARF}$/$p16^{INK4A}$/skin carcinogenesis/xeroderma pigmentosum. J Invest Dermatol 119:1355–1360, 2002***

Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder associated with a germline nucleotide excision repair (NER) defect (Jung, 1986). XP patients exhibit an extreme sensitivity to sunlight and an increased frequency of sunlight-induced skin cancers, mainly basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma (Kraemer *et al*, 1987). Seven XP complementation groups (A–G) have been distinguished (corresponding to mutations in distinct genes involved in NER), as well as a variant group (XP variant), which is deficient in the mutagenic polymerase η (Masutani *et al*, 1999). The *XPC* group is the most prevalent complementation group in Europe, United States, and North Africa. *XPC* cells are exclusively deficient in the NER system that corrects the ultraviolet (UV)-induced lesions of the nontranscribed strand ("global genome repair"), but are fully proficient in the transcription coupled repair able to remove specifically UV-induced lesions of the transcribed strand of active genes (Hanawalt, 1994). Much progress has been made in understanding the process of skin carcinogenesis in XP patients. p53 mutations are the most common genetic alterations, present in more than two-thirds of the tumors (Giglia *et al*, 1998). *XPC* knockout mice are highly predisposed to UVB radiation-induced skin cancer (Sands *et al*, 1995), which carry a high proportion of p53 mutations. More recently, a role for the *INK4a-ARF* locus, which maps to chromosome 9p21 has also been demonstrated in human and murine skin carcinogenesis. This locus gives rise to two alternative transcripts that encode two proteins, $p16^{INK4a}$ (exons 1α, 2, and 3) and $p14^{ARF}$ (exons 1β, 2, and 3), both of which are involved in the negative control of cell proliferation (Sharpless and DePinho, 1999). $p16^{INK4a}$ is a cyclin-dependent kinase inhibitor that can specifically inhibit progression through the G_1 phase of the replicative cycle in cells that express the retinoblastoma protein, by blocking cyclin-dependent kinase 4 (CDK4) from phosphorylating retinoblastoma protein (Serrano *et al*, 1993; Ruas and Peters, 1998). The alternative transcript, $p14^{ARF}$, in response to oncogenic signals such as *c-myc*, E1A, activated Ha-ras, and E2F1, specifically activates the p53 pathway, thereby inducing cell cycle arrest or apoptosis. Activation of $p14^{ARF}$ results in stabilizing p53 by preventing MDM2-p53 from undergoing ubiquitin-mediated degradation (reviewed in Sherr and Weber, 2000).

Substantial data now implicate the *INK4a-ARF* locus in the development of epithelial skin carcinoma. Deletions encompassing

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Reprint requests to: Nicole Basset-Seguin, INSERM U532, Hôpital Saint-Louis, 1 avenue C Vellefaux, 75010 Paris, France. Email: nbs@chustlouis.fr

Abbreviations: SCC, squamous cell carcinoma; BCC, basal cell carcinoma; WT, wild type.

this locus are common in SCC lines and in SCC (Loughran *et al*, 1994).

$p16^{ink4a}$ is inactivated during keratinocyte immortalization (Loughran *et al*, 1994; Munro *et al*, 1999). Moreover, nullizygous *INK4a-ARF* (–/–) or specifically ARF (–/–) mice are susceptible to the development of SCC (Serrano *et al*, 1996; Kamijo *et al*, 1997). UV-induced *INK4a-ARF* mutations have been detected in 12.5% of sporadic epithelial skin tumors (Soufir *et al*, 1999). XP skin carcinomas show a higher frequency of *INK4a-ARF* mutations (29% of XP associated skin tumors) that are often multiple and associated in the same tumors with p53 mutations (Soufir *et al*, 2000b). Little is known, however, about the timing of *INK4a-ARF* mutation during the multistep process of skin carcinogenesis. To gain insight into the role of this locus in skin carcinogenesis, we performed a molecular analysis of skin tumors, of different histologic nature or stage, arising in a single XP patient who displays an unusual phenotype. Strikingly, we found that this patient, in addition to belonging to the *XPC* group, carries a germline mutation on the $p16^{INK4A}$ gene. This is the first report of a *XPC* patient presenting a $p16^{INK4a}$ germline mutation. Among the tumors, only the BCC carried somatic mutations in *INK4a-ARF* and p53, suggesting that these modifications occurred late in tumor progression rather than the initiation stage.

MATERIALS AND METHODS

DNA repair analysis and complementation test Unexposed XP skin biopsies were cultured to establish diploid fibroblast lines and examined for DNA repair level (unscheduled DNA synthesis, UDS) after UVC irradiation as described previously (Sarasin *et al*, 1992). In order to confirm at the molecular level the diagnosis of XP, and to determine which of the seven XP genes involved in NER is deficient in our patient, we carried out genetic complementation of his skin diploid fibroblasts using infectious retroviral particles expressing the wild-type XP cDNA (Carreau *et al*, 1995; Zeng *et al*, 1997). The level of DNA repair of the patient was quantified by incorporation of tritiated thymidine (UDS) in the DNA of his skin fibroblasts after increasing UVC doses.

Analysis of the *XPC* group by western blotting analysis Protein was extracted in 8 M urea buffer as described (Sybert *et al*, 1985). Twenty micrograms of protein was run through a 6% acrylamide–sodium dodecyl sulfate electrophoresis gel and then transferred on to PVDF membrane (Amersham Pharmacia Biotech, Les Ulis, France). Membranes were saturated overnight in phosphate-buffered saline containing 5% nonfat dried milk. The rabbit polyclonal rabbit anti-*XPC* anti-serum was raised against a carboxy terminal peptide (amino acids 921–940) of the human *XPC* protein. Working dilution was 1/1000 in saturation solution. As control, membrane were rehybridized using a mouse monoclonal antibody to β-catenin (Clone 14, Transduction Laboratories, Lexington, KY). Blots were revealed using ECL reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Tumor samples, normal skin, and DNA extraction Eight skin neoplasms (four actinic keratosis, two microinvasive SCC, one basal cell carcinoma, and one atypical fibroxanthoma) from the XP patient were collected following surgical resection. One part was immediately fresh frozen in liquid nitrogen and stored at –80°C. A second part was formalin fixed and paraffin embedded, and histopathologic assessment was performed. A biopsy was also obtained of nonsun-exposed normal skin of this XP patient. DNA was prepared from frozen samples as previously described (Daya-Grosjean *et al*, 1993).

Single strand conformation polymorphism (SSCP) analysis Coding sequence and flanking intronic sequences of exon 1α, exon 1β, and exon 2 of the *INK4a-ARF* gene were analyzed by polymerase chain reaction (PCR)-SSCP. Primer sequences for exons 1α and 2 were previously described (Soufir Soufir *et al*, 1999, 2000a). Exon 1β was analyzed through two overlapping PCR products generated by using the following primers: P14F1 5′-TCAGGGAAGGGCGGGTGCG-3′; P14R1 5′-GCCGCGGGA TGTGAACCA-3′ (PCR product: 245 bp); P14F2 5′-GCCGCGAGT GAGGGTTTT-3′; and P14R2 5′-CACCGCGGTTATCTCCTC-3′ (PCR product 257 bp). Exons 4–9 of p53 gene were amplified using the same conditions as previously described (Soufir *et al*, 1999). Total PCR reaction volume was 20 μl, including 50–100 ng of genomic DNA template, 30 pmol of each primer, 0.5 UI (international unit) of Taq polymerase (Gibco-BRL) and 0.1 μl of [α^{33}P]deoxycytidine triphosphate (Amersham). Final $MgCl_2$ concentration was 1.5 mM for all PCR reactions. All reactions were supplemented with 10% dimethyl sulfoxide for optimal PCR amplification. PCR was processed with an annealing temperature of 60°C for the three studied exons of the *INK4a-ARF* gene, and 55°C for exons 4–9 of p53. For SSCP, PCR products were migrated on two 0.1 × TBE/Hydrolink MDE gels (FMC Bioproducts, Tebu, France), with either 8% glycerol at room temperature or without glycerol at + 4°C. Gels were run at 8 W, either 14 h at room temperature or 12 h at 4°C, dried, and exposed to autoradiography. The entire procedure was repeated at least twice for each sample. Shifted bands were cut out of the gel, and reamplified using the primers mentioned above.

Sequence analysis Products from 50 μl PCR reactions from reamplified shifted bands and tumoral genomic DNA were purified using a Microcon 100 (Amicon, Millipore, France) and sequenced using dye-labeled terminator on an automated sequencer 310 (Applied Biosystem, Applera, France) according to the manufacturer's instructions.

RESULTS

Clinical presentation A 12 y old boy from Algeria had typical clinical features of XP (i.e., pronounced photosensibility from the age of 3 y, erythema of photo-exposed skin, poikiloderma, cutaneous xerosis, photophobia, and bilateral conjunctivitis). Starting at the age of 4, multiple skin neoplasms appeared progressively on sun-exposed areas (actinic keratosis, SCC, and BCC).

At the age of 7, the patient presented with acute respiratory syndrome leading to the discovery of a T cell lymphoblastic lymphoma involving the anterior mediastinum, thymus, pericardium, and left pleura. Blood analysis showed 40×10^3 per ml lymphocytes with 50% blasts. Immunotyping revealed that these lymphocytes were $CD1a^+$, $CD4^+$, $CD7^+$, $CD8^+$, $HLADR^+$, and weakly $CD3^+$. All B markers were negative. A bone marrow analysis showed low cell counts without blasts. Highly carcinogenic drugs (i.e., antracyclins, alkyling agents, or cyclophosphamide) were considered contraindicated in this patient and a milder treatment regimen, including corticosteroids, Aracytin and Purinethol was started and well tolerated. The patient did not receive radiotherapy. After the initiation cure followed by several consolidation cures, the patient was considered in complete remission and has been stable since 1997.

At the age of 10, this patient had two unusual tumors: an atypical fibroxanthoma localized on the left ear and an epithelioid hemangioma of the neck that were surgically removed.

DNA repair deficiency A near to wild-type recovery of UV cell survival was observed solely after transduction of the *XPC* cDNA (**Fig 1**). In contrast, transduction of other XP cDNA (as exemplified in **Fig 2** using the *XPD* cDNA) did not result in the recovery of UV cell survival (**Fig 1**). The level of repair defect has been quantified by UDS after UVC irradiation of the diploid fibroblasts of the patient, as previously described (Sarasin *et al*, 1992). As shown in **Fig 2**, the UDS level is only 10–15% of our control normal fibroblast lines and similar to a control *XPC* fibroblast line.

This allowed us to conclude that our patient belongs to the *XPC* group. The patient's fibroblasts complemented by the wild-type *XPC* gene exhibit normal UDS (not shown).

Recovery of *XPC* protein expression in stable *XPC*-transduced *XPC* keratinocytes Our complementation data (**Figs 1 and 2**) strongly supported the appartenance of XP208VI to the *XPC* group. To support further these functional observations, protein extracts were prepared from WT (198VI), XP208VI, and *XPC*-transduced XP208VI fibroblasts. A

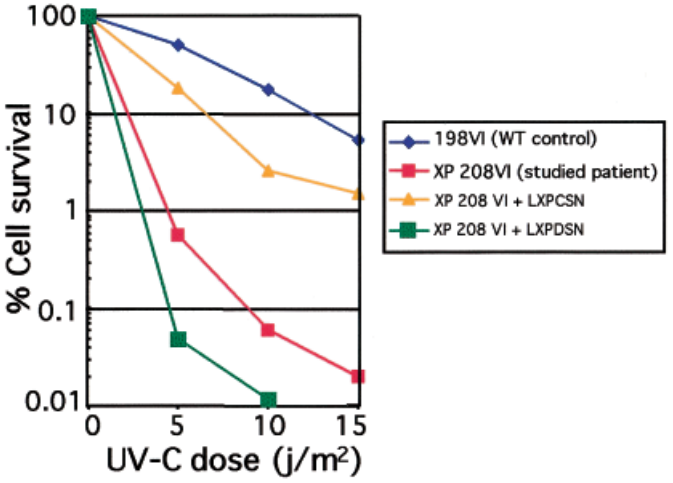

Figure 1. Genetic complementation and UV cell survival of the patient's skin fibroblasts. Relative cell survival is indicated as the ratio of the number of colonies in irradiated to nonirradiated cells. UVC doses are indicated. Note that transduction of the patient's fibroblasts (XP208 VI) by the *XPC* cDNA (XP208VI+L*XPC*SN) but not by the *XPD* cDNA (XP208 VI+LXPDSN) leads to near normal UV cell survival (198VI), thus assigning the patient to the XP complementation group C.

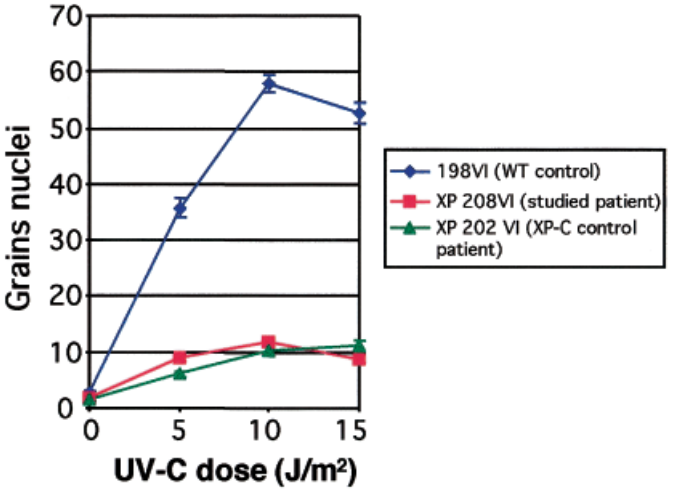

Figure 2. Severe alteration of DNA repair capacity of the patient's fibroblasts revealed by UDS after UVB irradiation. Number of autoradiographic grains over nuclei of the patient's and control fibroblasts were plotted as a function of the UVC dose. Note that the patient's fibroblasts (XP208 VI) and a control *XPC* (XP202 VI) strain reach a maximum of 10–15% of values obtained with normal cells (198VI).

keratinocyte strain, XP148VI, known to express a truncated *XPC* protein and its *XPC*-transduced counterpart (Arnaudeau *et al*, 2002), were used as control. Protein were processed for western blot analysis using the anti-*XPC* rabbit anti-serum (**Fig 3**). The apparently full length *XPC* protein (125 kDa) was detected in a WT control cell (198VI). In contrast, the *XPC* protein was neither detected in extracts from XP208VI nor, as already shown, in extracts from the *XPC* control cell strain XP148VI (Arnaudeau *et al*, 2002). In both XP cell strains (XP208VI, XP148VI) the absence of *XPC* protein detection indicated the absence of the *XPC* epitope recognized by the anti-serum raised against the carboxyterminal part of the *XPC* protein. On the contrary, *XPC*-transduced cells, i.e., XP208VI + *XPC* and XP148VI + *XPC*, expression of the

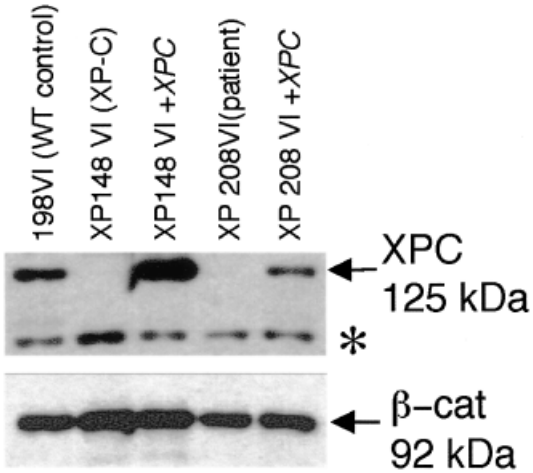

Figure 3. Absence of the full length *XPC* protein in XP208VI patient cells. Western blot analysis was carried out using protein extracts from XP208VI cells before XP208VI (*XPC*) and after retroviral transduction (XP208VI+*XPC*). 198VI (WT control) is a normal fibroblast strain. XP148VI (*XPC*) is a keratinocyte strain known to belong to the *XPC* complementation group. XP148VI+*XPC* are XP148VI cells following retroviral transduction of *XPC* (Arnaudeau *et al*, 2002). Note that *XPC* is not detected in extracts from XP148VI and XP208VI (studied patient), whereas the full length protein is re-expressed in transduced cells as in XP198 WT control. The same membrane was stripped and then rehybridized using anti-β-catenin monoclonal to control that similar amounts of protein were present in this area of the membrane. The star indicates a nonspecific band found in any condition.

apparently full length *XPC* protein was recovered as compared with that detected from WT 198VI extracts. As in most other *XPC* patients studied to date (Chavanne *et al*, 2000), the absence of the full length *XPC* protein in XP208VI cells could be considered as a definitive assignment to the *XPC* complementation group.

Mutational analysis of the *INK4a-ARF* locus SSCP analysis revealed abnormal bands for exon 1α only in one tumor (sample 7), the BCC. In exon 2, a common abnormal migration pattern was observed from all DNA analyzed (tumoral, normal skin, and normal fibroblast cell line), with an additional bandshift in tumor sample 7, the BCC. No abnormal band was seen for exon 1β of $p14^{ARF}$. DNA sequencing of abnormal bands revealed three mutations located within the cDNA sequence (**Table I** and **Fig 4**). One was characterized as a germline mutation, as it was present in DNA from all tumoral samples as well as from the patient's normal skin and fibroblast cell line. This germline mutation was a G>T transversion resulting in a mis-sense mutation in the $p16^{INK4a}$ reading frame (Ala127Ser). In contrast, this mutation had no consequence on $p14^{ARF}$, as it lies outside of its coding sequence (**Table I**). The two other alterations are somatic mutations of the BCC (tumor 7), which are characteristic UV-induced mutations occurring at dipyrimidic sites. One was a C:G>T:A transition located in the 5′ untranslated sequence of $p16^{INK4a}$ cDNA, possibly corresponding to a regulatory mutation. The second was a tandem CC:GG>TT:AA transition and corresponds to a known mutational hotspot in XP-associated skin tumors (Soufir *et al*, 2000b). This mutation also affects the $p14^{ARF}$ reading frame, at a codon conserved between mouse and human cDNA. The nucleotide composition of this codon (CCC) makes it a prime target for mutagenesis by UV.

Mutational analysis of the p53 gene SSCP analysis revealed a unique bandshift in exon 7 of the p53 gene, in BCC 7, corresponding to a tandem CC:GG>TT:AA transition that resulted in a mis-sense mutation at codon 248 of the p53

Table I. $p16^{INK4a}$, $p14^{ARF}$, and p53 mutations characterized in *XPC* skin epithelial tumors

Samples analysed	Histological Diagnosis	*INK4a-ARF* Sequence change	$p16^{INK4A}$ aa change	$p14^{INK4A}$ aa change	p53 sequence change	p^{53} aa change	Tumor
Skin tumors							
1	microinvasive SCC	tcGca > td**T**ca	Ala127Ser				lip
2	AK	tcGca > td**T**ca	Ala127Ser				forehead
3	AK	tcGca > td**T**ca	Ala127Ser				ear
4	AK	tcGca > td**T**ca	Ala127Ser				vertex
5	microinvasive SCC + AK	tcGca > td**T**ca	Ala127Ser				vertex
6	AK	tcGca > td**T**ca	Ala127Ser				vertex
7	nodular BCC	tcGca > td**T**ca	Ala127Ser		aaC Cgg > aaTTgg	Asn247Asn/ Arg248Trp	right eye
		gcCCgt > gc**TT**gt	Proll4Leu	Ala128 Ala/ Arg129Cys			
		gtGgg > gttg (−69 atg)	5′UTR/none				
8	Atpicalfibroxanthoma	tcGca > td**T**ca	Ala127Ser				ear
Normal skin		tcGca > td**T**ca	Ala127Ser				
Fibroblastline		tcGca > td**T**ca	Ala127Ser				

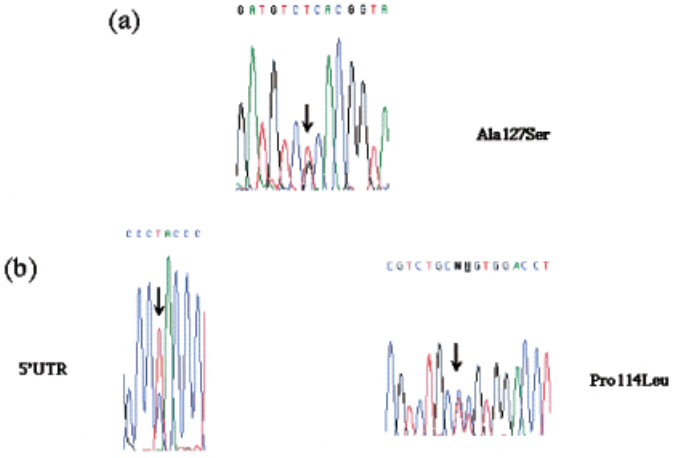

Figure 4. Germline and somatic mutations of the *INK4a-ARF* gene. Arrows above the automated sequence traces indicate the positions of mutated bases. The wild-type sequence and the nucleotide changes, and the coding effect of the changes on $p16^{INK4a}$ and $p14^{ARF}$ are listed in **Table I**. The sequence is shown 5′→3′ for the coding strand. The sequence is written 5′→3′ for the coding strand; the mutated base is written in upper case letters. The text in bold indicates nucleotide and amino acid changes. The text in italics, indicates the germline $p16^{INK4a}$ mutation. Mutations indicated by an asterisk are predicted to be UVB-induced mutations. Mutations on conserved residues of $p14^{ARF}$ are underlined.

gene (**Table I**). This mutation is a p53 hotspot found predominantly in skin cancers from XP patients (Beroud and Soussi, 1998).

DISCUSSION

To gain insight into the role of the *INK4a-ARF* and p53 genes in XP skin carcinogenesis, we performed a molecular analysis of the *INK4a-ARF* and p53 genes in skin tumors with different characteristics and stages arising in a single XP patient that displayed a particularly severe phenotype.

Remarkably, our XP patient developed three unusual tumors. To the best of our knowledge, non-Hodgkin lymphoblastic mediastinal lymphoma has never been described in a XP patient (Kraemer *et al*, 1987). Epithelioid hemangioendothelioma arising in the skin is extremely rare, and was also not previously described in XP patients (Kraemer *et al*, 1987). Atypical fibroxanthoma is a pleomorphic tumor usually classified as a distinct subtype of malignant fibrous histiocytoma that usually occurs on actinically damaged skin in the elderly, only rarely reported in XP patients (Patterson and Jordan, 1987; Dilek *et al*, 2000).

Unexpectedly, this patient was found to carry a $p16^{INK4a}$ germline mutation (Ala127Ser) characterized by its presence in normal skin and a fibroblast cell line as well as in all the tumor samples analyzed. Several arguments convey toward a pathogenic effect of this mutation. First, this mutation is located in the fourth ankyrin domain of the protein, a domain crucial in $p16^{INK4a}$ function. Secondly, this mutation was reported as a germline mutation in patients that developed different types of cancer: melanoma (Gruis *et al*, 1995); lung cancer (Okamoto *et al*, 1994); and primary human esophageal tumors (Zhou *et al*, 1994; Suzuki *et al*, 1995). Furthermore, in Suzuki *et al* (1995), this mutation was associated with loss of the normal remaining allele in the esophageal tumor. Ala127Ser was also characterized as an acquired somatic mutation in a SCC of the bladder (Gonzalez-Zulueta *et al*, 1995). Some functional studies did not find any effect of this variant on CDK4 binding (Koh *et al*, 1995; Ruas *et al*, 1999). A recent study, however, reported that testing of $p16^{INK4a}$ interactions with CDK in protein binding assays is an unreliable predictor of mutant $p16^{INK4a}$ function in cells (Becker *et al*, 2001). Indeed, in one study, this *Ala127Ser* allele was intermediate in its ability to cause growth cell arrest (Koh *et al*, 1995). Altogether, these data indicate that Ala127Ser may be a $p16^{INK4a}$ germline mutation rather than a polymorphism. In addition, our hypothesis that abrogation of $p16^{INK4a}$ could probably have contributed to the patient's particularly severe phenotype is reinforced by previous observations. First, germline defects of the *INK4a-ARF* locus might predispose to hematologic malignancies: (i) a germline mutation of $p16^{INK4a}$ that was recently shown to predispose a patient to multiple myeloma, which is another hematopoietic disorder (Dilworth *et al*, 2000), and (ii) mice nullizygous or heterozygous for the *INK4a-ARF* locus are highly prone to lymphoid malignancies as well as reticulosarcomas (Kamijo *et al*, 1997). Second, inactivation of the *INK4a-ARF* locus occurs commonly in human lymphoid disorders (Ruas and Peters, 1998) as well as in malignant fibrous histiocytoma, a tumor histologically related to atypical fibroxanthoma (Brinck *et al*, 1998; Simons *et al*, 2000).

Three somatic mutations were also detected, one in the p53 gene, and two in the *INK4a-ARF* gene, that were all found in only one of eight (12.5%) skin tumors. This is an overall low mutation frequency. This contrasts with the high frequency of p53

mutations detected in normal skin, precancerous, and cancer lesions arising in XP patients (Williams *et al*, 1998). This discrepancy may have several explanations. First, it should be noted that all but one tumor (85%) were small lesions; i.e., either precancerous lesions (actinic keratosis in 57% of cases) or microinvasive SCC (in 28% of cases). In line with this, in a previous study, XP skin tumors less than 8 mm in diameter were shown to harbor a significantly lower frequency of p53 mutation than larger tumors from the same XP patients (Matsumura *et al*, 1995). Additionally, our tumor was not microdissected and this could account for a lower sensitivity of mutation detection. Finally, in our particular case, the presence of a $p16^{INK4a}$ germline mutation could have decreased the need for additional p53 mutations.

Moreover, we previously detected *INK4a-ARF* mutations in only one of six sporadic SCC of early grade (Bowen diseases or actinic keratosis; (Soufir *et al*, 1999), which correlate with our present results and seem to signify that such mutations occur in the late stage of tumor development.

All three mutations detected in the BCC were characteristic of UV-induced lesions, occurring at dipyrimidinic sites, and located in two cases on the nontranscribed strand of the gene. The third mutation is due to an unrepaired DNA lesion that lies outside the transcribed sequence and therefore is not preferentially repaired. This mutational pattern is highly characteristic of *XPC* patients, proficient in preferential repair of active transcribed genes (Giglia *et al*, 1998; Soufir *et al*, 2000b). It is important to note that mutations of the three genes (p53, $p16^{INK4a}$, and $p14^{ARF}$) occurred in the same tumor. This association was previously found in XP tumors (Soufir *et al*, 2000b), and is probably related to the high genetic instability linked to the DNA repair defect in these patients. The accumulation of unrepaired mutations leads to the preferential selection of tumoral clones sharing inactivation of multiple tumor suppressor pathways.

In conclusion, we show for the first time that the coexistence of germline inactivation of a DNA repair gene and a cell cycle regulator gene that may have acted in concert to generate cancer proneness in a patient.

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